Case Report

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Successful mutation-specific chaperone therapy with 4-phenylbutyrate in a child with progressive familial intrahepatic cholestasis type 2

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Background & Aims: Progressive familial intrahepatic cholestasis type 2 (PFIC2) is due to mutations in *ABCB11* encoding the canalicular bile salt export pump (BSEP) of hepatocyte. Liver transplantation is usually required. 4-phenylbutyrate (4-PB) has been shown *in vitro* to retarget some selected mutated apical transporters. After an *in vitro* study in a hepatocellular polarized line, we tested 4-PB treatment in a child with a homozygous p.T1210P BSEP mutation.

Methods: Can 10 cells were transfected with plasmids encoding wild type Bsep ($Bsep^{wt}$) and mutated p.T1210P Bsep ($Bsep^{T1210P}$), both tagged with GFP. Then, cells were treated with 4-PB at 37 or 27 °C, immunostained and analyzed using confocal microscopy. The child received 4-PB orally in two divided doses and BSEP liver immunostaining was performed before and after 4-PB as well as bile analysis.

Results: In Can 10 cells, in contrast to $Bsep^{wt}$-GFP, $Bsep^{T1210P}$-GFP was not detected at the canalicular membrane but in the endoplasmic reticulum. 4-PB as well as incubation at 27 °C partially corrected $Bsep^{T1210P}$–GFP targeting to the canalicular membrane, while combined treatments resulted in normal canalicular localization. In the child, we showed that 4-PB improved clinical and biological parameters of cholestasis and liver function. Also, canalicular expression of p.T1210P BSEP mutant was partially corrected as was biliary bile acid excretion.

Conclusions: The results illustrate for the first time the therapeutic potential of a clinically approved chaperone drug in a selected patient with PFIC2 and support that bile secretion improvement might be due to the ability of 4-PB to retarget mutated BSEP.

Keywords: PFIC2; ABCB11; BSEP; Can 10.

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Abbreviations: 4-PB, 4-phenylbutyrate; BSEP/Bsep, bile salt export pump; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum associated degradation; GFP, green fluorescent protein; GGT, gamma-glutamyltransferase; LT, liver transplantation; PDI, protein disulfide isomerase; PFIC2, progressive familial intrahepatic cholestasis type 2; ZO-1, zonula occludens 1.

Introduction

Progressive familial intrahepatic cholestasis type 2 (PFIC2) is an autosomal recessive disease due to mutations in *ABCB11* encoding the bile salt export pump (BSEP) [1]. BSEP is localized at the canalicular membrane of hepatocytes and is the major transporter responsible for biliary bile acid excretion. In PFIC2, cholestasis usually appears within the first year of life. Impaired biliary bile acid excretion leads to a decreased bile flow, bile salt accumulation in hepatocytes, ongoing hepatocellular damage and increased risk of hepatocellular carcinoma. Patients develop severe pruritus, fibrosis, and end-stage liver disease before adulthood. Medical therapy with ursodeoxycholic acid (UDCA), rifampicin, and surgical therapy such as biliary diversion may provide some symptomatic relief. Nevertheless, in the majority of cases, liver transplantation (LT) is required because of unremitting pruritus, hepatic failure or hepatocellular carcinoma [1]. In the liver of most children with PFIC2, BSEP is not detected at the canaliculus [1]. Involved mutations lead to premature protein truncation and/or failure of protein production, but missense mutations are also common defects [1]. *In vitro* studies showed that some missense mutations affect protein processing/trafficking causing retention in the endoplasmic reticulum (ER) and subsequent endoplasmic reticulum associated degradation (ERAD) [2–6]. 4-phenylbutyrate (4-PB), a clinically approved pharmacological chaperone [7], has been shown *in vitro* to retarget at the canalicular membrane some mislocalized Bsep mutants [3,4], as well as other mutated apical transporters [8]. Herein, we report on the first experience of 4-PB therapy in a patient with PFIC2 carrying a homozygous *ABCB11* missense mutation (p.T1210P) leading to mistargeting and intracellular retention of the BSEP mutant. We

show that 4-PB therapy improves clinical and biological parameters of cholestasis and liver function. Furthermore, canalicular expression of p.T1210P BSEP mutant was partially corrected as was biliary bile acid excretion. 4-PB was also able to partially retarget at the canalicular membrane this mutant introduced in the hepatocellular line Can 10.

Patients and methods

Case report

General information regarding the patient girl has been previously reported (see family 12) [1]. She was diagnosed with PFIC2 before 1 year of age. The p.T1210P mutation was identified in both alleles of *ABCB11* and BSEP liver immunostaining was negative. Despite optimal treatment (UDCA: 600 mg m^{-2} of body area per day, rifampicin: 20 mg kg^{-1} d^{-1}, external biliary diversion) [9], severe cholestasis persisted with intractable itching, and she developed liver failure. Biliary diversion was closed because it was not efficient. She was enlisted for LT at age 10 years. Treatment with 4-PB was started at age 10 years as a rescue option after an *in vitro* study showed some rationale for this therapy (see Results section). UDCA and rifampicin were maintained without dosage change during 4-PB therapy.

In vitro studies

Cell culture, mutagenesis, transfection, and treatment

Can 10, a rat hepatocellular polarized line, that expresses only minimal level of Bsep was used. Cells were cultured as previously described [10]. Patient's mutation p.T1210P was introduced in a plasmid encoding a rat Bsep-green fluorescent protein (GFP) fusion protein [3], with the following primer forward 5′-GCTCCCAGAGAAATATGAACCTAATGTTGGGATCCAGGGC-3′, using QuickChange Lightning Site-Directed Mutagenesis kit (Stratagene, USA) according to manufacturer's instructions. Rat Bsep plasmid was used because of the high degree of homology with human BSEP and the higher level of expression in cultured cells [2]. Successful mutagenesis was verified by sequencing of the whole construct. Can 10 cells, seeded onto glass coverslips (1.5×10^4 cm^{-2}) were transiently transfected at day 3 of culture with $Bsep^{wt}$-GFP or $Bsep^{T1210P}$-GFP plasmid, using Fugene HD (Roche, Diagnostics, Meylan, France) according to the manufacturer's instructions. 24 h after transfection, cells were treated with or without 1 mM 4-PB (Biovision, clinisciences, Montrouge, France, diluted in PBS) during 24 h at 27 °C or 37 °C.

Immunofluorescence

Cells were fixed and immunostained (ice-cold 2% formaldehyde 1 min, then 100% methanol 10 min). Primary antibodies used for immunostaining were as follow: mouse anti-GFP (Roche Diagnostics, Meylan, France) (1/80), rabbit anti-GFP (Abcam, Cambridge, UK) (1/120), rat anti-zonula occludens 1 (ZO-1) (anti-ZO-1 was a gift from B.R Stevenson, Edmonton, Canada, and was used undiluted), and mouse anti-protein disulfide isomerase (PDI) (EnzoLifeSciences, Villeurbanne, France) (1/300); and they were incubated 1 h at 37 °C. Cells were rinsed, then incubated for 15 min at 37 °C with the appropriate Alexa-conjugated secondary antibodies (Molecular Probes, Oregon, USA) (1/500). The coverslips, mounted in mounting media (Sigma–Aldrich Chimie, Lyon, France), were examined, two days after transfection in Can 10 cells, with a confocal microscope (Eclipse TE-2000-Nikon-C1) equipped with a 60x objective and xy optical sections were taken in 0.35 µm steps. Bsep-GFP levels were quantified on confocal sections, using imageJ software.

Patient treatment with 4-phenylbutyrate

4-PB therapy was started at a daily dose of 200 mg kg^{-1} d^{-1} divided in 2 oral doses of sodium phenylbutyrate (AMMONAPS, Swedish Orphan Inter AB). Patient was followed prospectively. In order to get the best effect, the dose was increased up to a maximum of 500 mg kg^{-1} d^{-1}, as recommended [7,11]. Before starting on 4-PB, written informed consent was obtained from the family, according to the guidelines of the local ethical committee.

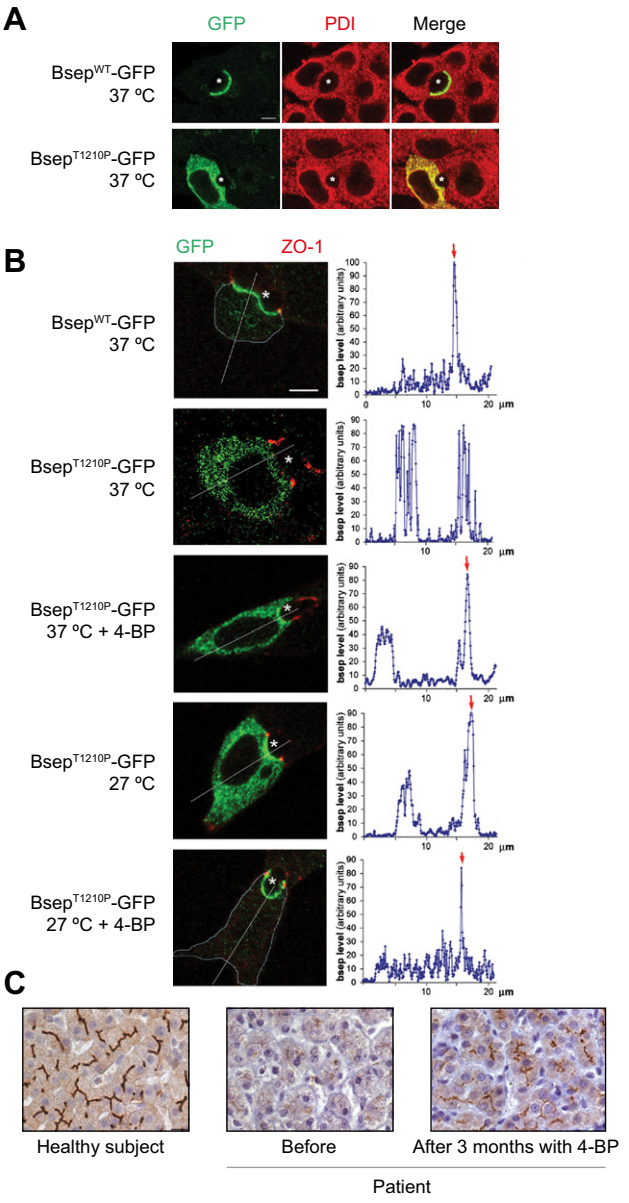

Fig. 1. Studies of $Bsep^{T1210P}$–GFP in hepatic polarized Can 10 cells and effect of 4-PB on $BSEP^{T1210P}$ in a PFIC2 child. (A) Immunolocalization of $Bsep^{wt}$-GFP, $Bsep^{T1210P}$–GFP (green) and protein disulfide isomerase (PDI) (red). (B) 4-PB and low temperature effects on $Bsep^{T1210P}$–GFP (green) immunolocalization. Tight junction protein zonula occludens 1 (ZO-1) immunostaining revealed a canalicular joint (red). Bsep–GFP levels were quantified from left to right along the white line indicated on the corresponding confocal section. Resulting graphs are shown on the right. Red arrows indicate canalicular fluorescence reinforcement, when present. A thin blue-grey line was drawn on confocal images to delineate cell limits, when they were hardly visible. * canaliculus; bar: 5 µm. (C) BSEP immunostaining of human liver sections: healthy subject (left), patient before (middle) and after 3 months with 4-PB therapy (right). Bar: 30 µm. (This figure appears in colour on the web.)

Itching evaluation

Itching was quantified by the medical staff and the parents using a visual analog scale (graduated from 0 to 10), during day time and night time. Each presented score corresponded to the mean of these evaluations. A score of 0 indicated no